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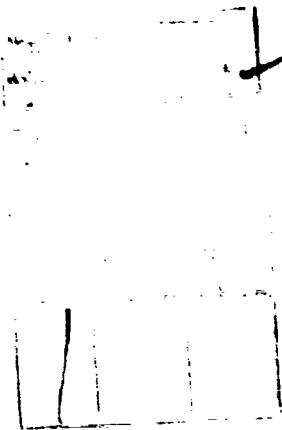BIOCHEMICAL STUDIES ON THE TOXIC NATURE OF SNAKE VENOM

by

Chen-chung Yang, D. Med. Sc.

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Republic of China

June 1969

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REPORT No. J-262-6 (Final Report No. 3)

GRANT No. DA-CRD-AFE-S92-544-68-G124

DA Project/Task/Work Unit No. 2N061102B71D   00 063FE

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Studies on the chemical properties of cobrotoxin have established its amino acid composition, and revealed that the toxin has a single peptide chain of 62 amino acids cross-linked by 4 disulfide bonds with amino terminal leucine and carboxyl terminal asparagine. In comparison with the entire sequence of toxin α from the venom of Naja nigricollis the similarities obviously must lie in its alike chemical structure. Knowledge of the sequence of cobrotoxin is required in order to understand the location of the essential amino acid residues required for the maintenance of the active conformation of this type. It is the purpose of this experiment to present the sequence of amino acids in cobrotoxin.

Reduced and S-carboxymethylated cobrotoxin was digested with trypsin and chymotrypsin. Nine peptides were isolated from the tryptic digest by a combination of electrophoresis and chromatography on paper. The amino acid sequence in these peptides was determined by both the application of the Edman degradation and the use of proteolytic enzymes, leucine aminopeptidase and carboxypeptidases. The arrangement of nine peptides into a linear structure was made by comparing the amino acid compositions of the overlapping peptides isolated from chymotryptic digest of RCM-cobrotoxin. The striking distributional regularities of the basic and hydrophilic residues in the sequence, and the importance of the positions of half-cystinyl residues which form the disulfide bonds for maintaining the protein in its active configuration are considered in connection with structure-activity relations of the protein.

ABSTRACT

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Studies on the chemical properties of cobrotoxin have established its amino acid composition, and revealed that the toxin has a single peptide chain of 62 amino acids cross-linked by 4 disulfide bonds with amino terminal leucine and carboxyl terminal asparagine. In comparison with the entire sequence of toxin α from the venom of Naja nigricollis the similarities obviously must lie in its alike chemical structure. Knowledge of the sequence of cobrotoxin is required in order to understand the location of the essential amino acid residues required for the maintenance of the active conformation of this type. It is the purpose of this experiment to present the sequence of amino acids in cobrotoxin.

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In comparison with the entire sequence of toxin α and erabu-toxin b, 31 residues are found to be common to the three neurotoxins. It is noteworthy that all cystinyl residues which are important to the biological activity in neurotoxin are all in the same position, and leave the center sequence from residue 25-40 free. It is speculated that this central non-crosslinked sequence containing most of the basic and aromatic amino acids in close order might be the "active site" of the neurotoxin molecule. Moreover, the sequence of cobrotoxin shows very closely related to that of toxin α isolated from the same family of snake. There are only teen differences in amino acid composition of toxin α which express themselves in 12 different residues and one missing residue along the whole chain. In view of the general similarity among neurotoxins isolated from different snake venoms, investigations of the structural differences among them should reveal a good deal about the structural features responsible for the venom toxicity.

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The Amino Acid Sequence of Cobrotoxin

I. Introduction

Studies on the chemical properties of cobrotoxin have established its amino acid composition, and revealed that the toxin has a single peptide chain of 62 amino acids cross-linked by 4 disulfide bonds with amino terminal leucine and carboxyl terminal asparagine (1,2). In comparison with the entire sequence of toxin α from the venom of Naja nigricollis (3) the similarities obviously must lie in its alike chemical structure. Knowledge of the sequence of cobrotoxin is required in order to understand the location of the essential amino acid residues required for the maintenance of the active conformation of this type. It is the purpose of this experiment to present the detailed results which lead to the formulation of the sequence of amino acids in cobrotoxin.

As a prerequisite for this study reduced and S-carboxymethylated cobrotoxin was digested with trypsin and chymotrypsin. The resulting peptides were separated and their amino acid sequences were determined largely through the application of the Edman degradation technic. From the results of this investigation, the 62 amino acid residues of cobrotoxin have been placed in sequence.

II. Materials and Methods

Cobrotoxin used in this study was prepared from Formosan cobra (Naja naja atra) venom as previously described (4). Trypsin was the TPCK-treated crystallized preparation, Lot 7 KA; α-chymotrypsin, the three times crystallized salt-free preparation, Lot 6 JF; leucine aminopeptidase, the DFP treated suspension, Lot 6 IC; and carboxypeptidases A and B, the DFP treated suspensions, Lot 6 FC and Lot 29 respectively. These enzyme preparations were all the products of Worthington Biochemical Corp. Reagent grade of β-mercaptoethanol and iodoacetic acid obtained from Matheson and Coleman Company and purified phenylisothiocyanate obtained from Pierce Chemical Company were used. All other reagents were of analytical grade. Sephadex G-25 was purchased from Sigma Chemical Company and urea was a Mallinchrodt reagent. Pyridine was distilled prior to use.

1. Reduction and alkylation of cobrotoxin

Reduction and alkylation of cobrotoxin was performed according to the method described by Crestfield, Moore and Stein (5). Three hundred mg of cobrotoxin was dissolved in 10 ml of 0.2 M Tris-HCl buffer (pH 8.2) containing 8 M urea and 0.3 ml of β-

mercaptoethanol was added. The glass-stoppered tube was flushed with N_2 and left at room temperature for 4 h. For alkylation, a freshly prepared solution of 900 mg iodoacetic acid in 2.3 ml of 2 N NaOH was added with stirring and the pH of the solution maintained at pH 8.6 with the aid of 2 N NaOH. After 30 min the solution was desalted by passing through a column of Sephadex G-25. The reduced and S-carboxymethylated (RCM-) cobrotoxin emerged in the void volume was pooled and lyophilized.

2. Tryptic digestion of RCM-cobrotoxin

The RCM-cobrotoxin was dissolved in 0.1 M NH_4HCO_3, pH 8.2, to make a 1 % solution and a sufficient trypsin solution (10 mg/ml in water) was added to give a protein : enzyme ratio of 50 : 1. After incubation at 37° for 2 h, the reaction was stopped by the addition of 1 M acetic acid to pH 4 and the digest was dried over P_2O_5 in a desiccator under vaccum.

3. Fingerprinting

The dried digest was dissolved in water and aliquots corresponding to 1 mg of the original protein were subjected to electrophoresis at pH 5.4 with pyridine-acetic acid-water (20 : 7 : 973, v/v) (6) on 74 x 56 cm sheets of Whatman No. 3 MM paper. The electrophoresis was carried out in a varsol cooled tank with a potential gradient of 51 V/cm for 60 min. After drying, chromatography was run with n-butanol-acetic acid-water-pyridine (15 : 3 : 12 : 10, v/v) (7) by the descending method for 18 h at room temperature. Peptides on the map were developed initially with 0.2 % ninhydrin in absolute ethanol and thereafter with the Ehrlich reagent (8) to characterize peptides containing tryptophan.

4. Isolation of peptides

The tryptic peptides from 60 mg digested protein were separated by paper electrophoresis at pH 5.4 on a sheet of 74 x 30 cm as described above. After which, 1 cm strips were cut from both sides of the electrophoretogram and developed with 0.2 % ninhydrin in acetone (w/v). Using the developed strips as markers the areas of the electrophoretogram containing the peptides were cut out and stitched to fresh sheets of Whatman No. 3 MM paper for descending chromatography as described above. Marker strips were cut out and developed to detect the positions of the peptide bands which were then cut out, eluted with 1 N acetic acid and dried. In some cases, an additional electrophoretic separation using pH 1.9 with 88 % formic acid-acetic acid-water (25 : 87 : 888, v/v) (9) at 55 V/cm was necessary to obtain the purified peptides.

5. Chymotryptic digestion of tryptic peptides

About 1 μ mole of the tryptic peptide was dissolved in 1 ml of 0.1 M NH_4HCO_3, pH 8.2 and 0.1 ml of chymotrypsin solution (5 mg/ml) was added. The mixtures were incubated at room temperature for 24 h. After which the pH of the solution was lowered and the solution was taken to dryness for fingerprinting and for isolation of peptides as described above.

6. Amino acid analysis

For the determination of amino acid compositions, the peptide bands containing 0.2-0.5 μ mole were eluted with constant boiling 5.7 N HCl, and hydrolyzed at 110° for 24 h in evacuated, sealed tubes. Amino acids were determined on a Technicon amino acid Autoanalyzer with norleucine as an internal standard or identified by paper electrophoresis at pH 1.9. Analyses for the peptides containing tryptophan were made after complete hydrolysis of the peptide with leucine aminopeptidase in 0.1 M NH_4HCO_3 buffer, pH 8.2.

7. Determination of N-terminal sequence using the Edman degradation

The sequence of amino acid residues in peptides was determined primarily by means of the Edman PTH procedure. The following modification of the method by Doolittle (10), and by Light and Groenberg (11) was routinely used. About 0.5 -1 μ mole of dried peptide specimen in a glass-stoppered centrifuge tube was dissolved in 1 ml of 0.4 M dimethylallylamine in 60 % pyridine buffer to which the pH has been adjusted to 9.5 with 1 N trifluoroacetic acid. 40 μl of phenylisothiocyanate was added and the tube was flushed with pure N_2 gas for about 10 sec. The mixture was then incubated at 40° for 1 h. After which the excess reagent was removed thoroughly by repeated extractions with 2 volumes of benzene 5 times and cyclohexane 3 times. The aqueous phase was flushed with a stream of N_2 gas to remove the traces of organic solvent and then taken to dryness in a dessicator containing P_2O_5 and NaOH pellets under reduced pressure. Cyclization was performed at 40° for 1.5 h in 0.2 ml of glacial acetic acid-conc. HCl (5 : 1) under N_2. The cyclized mixture was taken to dryness, dissolved in 0.5 ml of 0.01 N HCl and extracted 3 times with an equal volume of ether. The ether phase was evaporated to dryness and the PTH-amino acid was identified with the descending paper chromatography of Sjöquist (12) and of Edman and Sjöquist (13). Solvent A, C and F were used almost exclusively.

8. Determination of N-terminal sequence using leucine aminopeptidase (14)

The peptides about 0.1 μ mole were dissolved in 0.1 ml of water and the pH brought to 8.4 by the addition of 0.02 ml of 0.5 M NH$_4$HCO$_3$. 0.01 ml of leucine aminopeptidase solution (1 mg/ml in 0.4 mM MgCl$_2$) was added and the reactions were allowed to proceed at 38°. Aliquots (0.02 ml) of the digests were taken after ¼, ½ and 1 h, acidified with acetic acid and dried. The amino acids released were identified by paper electrophoresis at pH 1.9 and blank digestions were always run at the same time.

9. Determination of C-terminal sequence using carboxypeptidases (14)

The peptides were dissolved in 0.2 ml of 0.1 M NH$_4$HCO$_3$, pH 8.2, and 0.01 ml of carboxypeptidase B solution (1 mg/ml in water) and of carboxypeptidase A solution (1 mg/ml in water) were added. Digestions were carried out at 30° for 1 h, after which the digests were acidified and dried. The amino acid liberated were identified by paper electrophoresis at pH 1.9 and blank digestions were always run at the same time.

10. Detection of amide group in peptides

After prolonged treatment of acetic acid-conc. HCl in the Edman degradation of peptides, glutamine appeared mainly as PTH-glutamic acid, so that additional determination of the amide group was necessary. In most cases, the presence of amide group in peptides was determined by 24 h digestion of peptides with leucine aminopeptidase or carboxypeptidases. Sometimes the electrophoretic mobility of peptides measured at pH 5.4 indicating the net charge on the peptide was used for judging the presence of free carboxyl or its amide group.

11. Non-enzymatic cleavage of the peptide bonds

As has been mentioned by Narita and Titani (15), the use of the conc. HCl was helpful to overcome the difficulty in determination of the threonine and serine due to the lower recoveries of their PTH derivatives in the Edman degradation using acetic acid-conc. HCl mixture. This was performed with conc. HCl at room temperature overnight to cleave the acylserine and acylthreonine linkages in confirming the position of seryl and threonyl residues in the sequence of peptides.

12. Chymotryptic digestion of RCM-cobrotoxin and the separation of the chymotryptic peptides

One per cent of the RCM-cobrotoxin in 0.1 M NH$_4$HCO$_3$ (pH 8.2) was digested with chymotrypsin at a protein : enzyme ratio of 50 : 1 at 37° for 24 h. After which the pH of the solution was lowered and the digests were dried as described above. Fingerprints were

made, and the peptides were stained with 0.02 % ninhydrin (w/v) in absolute ethanol and developed at 60° for 20 min. The ninhydrin positive spots were cut out, washed, eluted with 5.7 N HCl and hydrolyzed at 110° for 24 h. In some cases, additional purification of the peptides using paper electrophoresis at pH 1.9 were necessary to give better analysis.

III. Results

1. Fingerprinting of tryptic peptides from RCM-cobrotoxin

The pattern of fingerprinting of tryptic peptide obtained by two dimensional paper electrophoresis and chromatography is shown in Fig. 1. Nine major spots (solid cycle) and 6 minor spots (broken cycle) are obtained and numbered orderly from the acidic to the basic side and on the basis of chromatographic mobility. Peptides T-4, T-5, T-7 and T-12 gave initially a yellow ninhydrin color indicating amino terminal glycine or threonine. Peptide T-2 gave only a faint brown ninhydrin color presumably because of the amino terminal asparagine or isoleucine (16). Only two peptides, T-8 and T-13 showed a positive reaction with Ehrlich reagent.

2. Amino acid composition of tryptic peptides

The amino acid composition together with the yields of the tryptic peptides from RCM-cobrotoxin is presented in Table I. Amino acid analyses were performed on acid hydrolysates, except peptides T-8 and T-13 were on their leucine aminopeptidase hydrolysates. Since, peptide T-1 has not been isolated in pure state, its amino acid composition was derived from the data that included 10 % of peptide T-10.

The amino acid composition of all peptides is compatible with their electrophoretic mobility and with chromatographic movement. Of the peptides obtained in yields of more than 18 %, all but one contain lysyl or arginyl residue at the carboxyl end at which trypsin is known to cleave the peptide bond. The exception is peptide T-1, which probably represents the carboxyl terminal sequence of the protein. Of the peptides obtained in yields of less than 6 %, two do not contain lysyl or arginyl residue but contain tyrosyl residue which is susceptible to chymotryptic digestion. Because of their unique composition, these peptides are clearly the fragment of peptides obtained in higher yield. Other peptides in lower yield show a composition that is exactly the sum of the compositions of two other peptides obtained in higher yield, indicating the bond less susceptible to trypsin.

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Thus, only the peptides obtained in higher yield account for the entire amino acid composition of cobrotoxin which is the following residues: Asp_8, Thr_8, Ser_4, Glu_7, Pro_2, Gly_7, Cys_8, Val_1, Ile_2, Leu_1, Tyr_0, Lys_3, His_2, Arg_6, Trp_1.

### 3. Fingerprinting of chymotryptic peptides from T-3

The pattern of the peptide map is shown in Fig. 2 where 5 main spots are obtained. Peptide T-3-C-1 gave initially a yellow color, and peptide T-3-C-3 initially brown color indicating serine is probably the amino terminal.

### 4. Amino acid composition of chymotryptic peptides from tryptic peptide T-3

The amino acid composition of the chymotryptic peptides from T-3 is presented in Table II. Although peptide T-3 contains only one tyrosyl residue which forming peptide bond susceptible to chymotryptic hydrolysis, 5 distinct peptides were obtained. Since T-3-C-4 is a dipeptide (see below), the total residues in the chymotryptic peptides agree well with the entire amino acid composition of T-3.

5. Amino acid sequence of the peptides

The half arrows above and under the abbreviations depict the results of enzymic digestion and of the successful applications of the Edman degradation, respectively.

T-1   H-CM-cys-Asn-Asn-OH
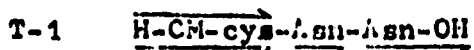
The sequence was established by the Edman degradation which yielded PTH-CM-cys, PTH-Asn and PTH-Asn subsequently. Digestion with leucine aminopeptidase for 1 h released only CM-cysteine, for 24 h gave CM-cysteine and asparagine. Since this tripeptide is the only peptide in tryptic digest which contains no lysyl or arginyl residue, it might be the carboxyl terminal sequence of the protein.

T-2   H-Asn-Gly-Ile-Glu-Ile-Asn-CM-cys-CM-cys-Thr-Thr-Asp-

Arg-OH
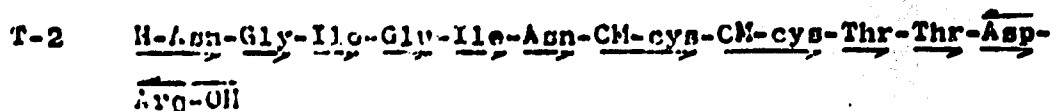
The Edman degradation was performed up to the 11th step successfully. Leucine aminopeptidase did not liberate any free amino acids, the true cause is not known. Digestion with carboxy-peptidases liberated arginine and aspartic acid subsequently. The glutamic acid at 4th position is not glutamine, because the peptide C-8 (H-Gly-Ile-Glu-Ile-Asn-OH) isolated from the chymo-

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tryptic digest of RCM-cobrotoxin (Table IV) has one net negative
charge and since PTH-Asn was detected at 6th step in this peptide
by the Edman degradation.

T-3   H-Leu-Glu-CM-cys-His-Asn-Gln-Gln-Ser-Ser-(CM-cys$_1$, Asp$_1$,

Thr$_5$, Ser$_1$, Glu$_2$, Pro$_1$, Gly$_3$)-CM-cys-Tyr-Lys-OH
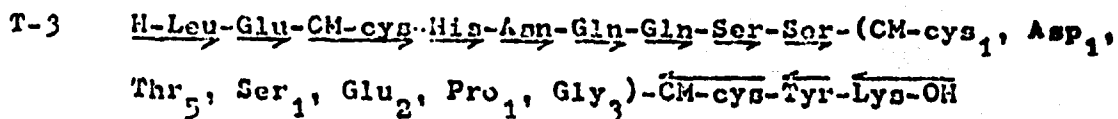
The amino acid composition of T-3 indicates that it contains
26 residues including leucine which is the only one solely present
in and appears as the amino terminal residue of the protein (1).
Thus, T-3 represents the amino terminal sequence of the protein
extended up to 26th amino acid residue.   The partial sequence was
elucidated by the Edman degradation, and with carboxypeptidases
which liberated lysine, tyrosine and CM-cysteine subsequently.

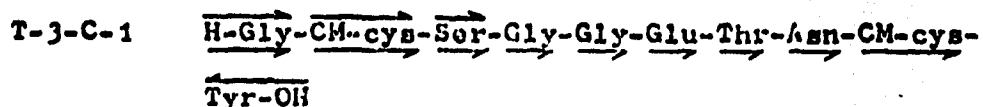T-3-C-1   H-Gly-CM-cys-Ser-Gly-Gly-Glu-Thr-Asn-CM-cys-

Tyr-OH

The sequence of the peptide was established by the Edman
degradation.   Carboxypeptidase A liberated only tyrosine.
Digestion with leucine aminopeptidase released Glycine, CM-cysteine
and serine subsequently.   24 h digestion with leucine aminopeptidase
gave free amino acids including glutamic acid but no aspartic acid.

T-3-C-2   H-Leu-Glu-CM-cys-His-Asn-Gln-Gln-OH

The sequence of the peptide was elucidated by the Edman
degradation.   Carboxypeptidase A released glutamine but no
glutamic acid.   1.5 h digestion with leucine aminopeptidase
liberated leucine and glutamic acid.   24 h digestion gave free
amino acids but with no aspartic acid.

Conc. HCl

T-3-C-3   H-Ser-Ser-Gln-Thr-Pro-Thr-OH
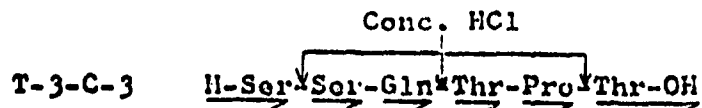
The sequence was established by the Edman degradation.
Since this peptide is neutral, glutamyl residue must be amidated.
24 h digestion with leucine aminopeptidase liberated only serine
and glutamine, and carboxypeptidase A did not liberate any free
amino acids.   The peptide was treated with conc. HCl at room
temperature for 16 h.   The products were separated by paper
electrophoresis at pH 1.9 into 2 major fractions in addition to
several minor bands.   One of the major bands contained serine
and glutamic acid, and the other contained threonine and proline.

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These confirmed that prolyl residue is at 5th position.

T-3-C-4   H-Thr-Thr-OH

The peptide gave an initially brownish yellow ninhydrin color and yeilded only threonine on amino acid analysis. It ran slightly faster than free threonine on the chromatogram using the solvent system of peptide mapping (n-butanol-acetic acid-water-pyridine, 15 : 3 : 12 : 10) and ran closer to isoleucine on the electrophoretogram at pH 1.9. On Technicon amino acid Autoanalyzer it emerged near the position of alanine. The analyses of hydrolyzed and unhydrolyzed aliquots of equal portions of the peptide indicated a double increase in ninhydrin color, suggesting that it is a dipeptide of threonylthreonine. Since no authentic specimen of threonylthreonine to compare with, the chromatographic and electrophoretic mobilities of this peptide remains to be explained.

T-3-C-5   H-Lys-OH

Since peptide T-3 contains only one lysyl residue, this peptide is present in the form of free lysine rather than polymer.

Conc. HCl

T-4   H-Gly-CM-cys-Gly-CM-cys-Pro-Ser-Val-Lys-OH
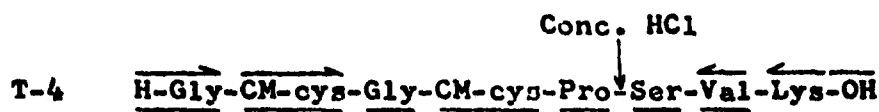
The Edman degradation established the above sequence. Leucine aminopeptidase and carboxypeptidases released free amino acids as indicated. On the treatment with conc. HCl at room temperature for 16 h, 2 major peptides in addition to 2 minor were obtained by paper electrophoresis at pH 1.9. One of the major bands contained serine, valine and lysine, and the other glycine, CM-cysteine and proline.

T-5   H-Thr-Glu-Arg-OH
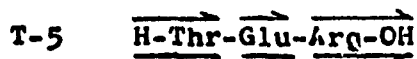
The peptide is neutral and gave initially a yellow ninhydrin color, indicating the presence of a glutamyl residue and an amino terminal threonine. This was confirmed both by the Edman degradation and by leucine aminopeptidase digestion.

T-6   H-Asp-His-Arg-OH
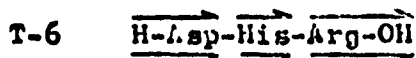
The sequence was elucidated both by the Edman degradation and by leucine aminopeptidase digestion.
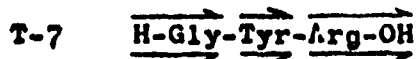
T-7   H-Gly-Tyr-Arg-OH

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This peptide gave initially a yellow ninhydrin color, indicating that glycine is the amino terminal. This was confirmed both by the Edman degradation and by leucine aminopeptidase digestion.

    T-8   H-Trp-Arg-OH
           ───→ ───→

The sequence was confirmed by the Edman degradation.

    T-9   H-Lys-Arg-OH
           ───→ ───→

The sequence was determined by the Edman degradation.

    T-10   H-Asn-Gly-Ile-Glu-Ile-Asn-CM-cys-CM-cys-Thr-Thr-Asp-

           Arg-CM-cys-Asn-Asn-OH

The composition of this peptide is the sum of the residues in T-1 and T-2. Since T-1 is the carboxyl terminal peptide, the above sequence of T-10 is deduced.

    T-11   This peptide has the same composition as T-3 except that it lacks the carboxyl terminal lysyl residue. The release of lysyl residue is probably due to the presence of tyrosine in a position amino terminal to lysine, forming a bond susceptible to chymotryptic digestion.

    T-12   H-Gly-Tyr-OH

This peptide gave initially a yellow ninhydrin color, indicating that glycine is the amino terminal. The lower yield and the unique composition of this peptide indicates that it is derived from the amino terminal part of the peptide T-7.

    T-13   H-Trp-Arg-Asp-His-Arg-OH

The composition of this double tryptic peptide is the sum of the residues in peptides T-6 and T-8. The resistance of the bond to trypsin indicates that asparyl residue is linked to arginyl residue.

    T-14   H-Lys-OH

    T-15   H-Arg-OH

Electrophoretic and paper chromatographic mobilities of these peptides indicate that both are present in free forms. The presence of free lysine and free arginine is presumbly derived from peptides T-3 and T-7.

6. Fingerprinting of chymotryptic peptides from RCM-cobrotoxin and their amino acid compositions

In an attempt to obtain the overlapping peptides, the RCM-cobrotoxin was digested with chymotrypsin. The pattern of finger-printing obtained is shown in Fig. 3. Since most of the tryptic peptides contain specific amino acid residue such as leucine, isoleucine, histidine, tyrosine, proline and tryptophan which can be used as marker, it is not difficult to link each other by analyzing the amino acid composition of the major peptides. The results are presented in Tables III and IV.

IV. Discussion

In the present studies, the specificity of trypsin has come to be anticipated. The variable yields of the tryptic peptides clearly indicate that the rate of hydrolysis of various bonds is different. The presence of aspartic acid in a position amino terminal or carboxyl terminal to arginine markedly decreases the rate of hydrolysis. This has occurred in peptides T-1 and T-2, and also in peptides T-8 and T-6. Longer hydrolysis splits the more resistant bonds but it has also resulted in hydrolysis of a bond carboxyl terminal to tyrosine as in peptides T-3 and T-7, probably due to the contaminating chymotrypsin in trypsin preparation, or the intrinsic action of the trypsin.

Chymotrypsin has shown far less specific in its action. In addition to the expected hydrolysis of bonds that are linked with tyrosine, tryptophan, asparagine, histidine and lysine, the hydrolysis of bonds that are carboxyl terminal to glutamine, threonine and aspartic acid has been observed. The peptides produced were obtained unexpectedly in higher yields.

Leucine aminopeptidase has proved to be useful in the determi-nation of the peptide containing glutamine, asparagine or tryptophan, and in the confirmation of the position of prolyl residue in the peptides which is in accord with the result obtained by Schroeder (17).

The destruction of serine, threonine, tyrosine and CM-cysteine in acid hydrolysis has commonly been observed. That of serine, and of threonine is between 5-10 %, that of CM-cysteine is between 20-40 %, that of tyrosine is somehow more variable.

The Edman degradation used in this study has shown to be satisfactory in determination of at least 11 residues. Serine and threonine were found to form the PTH derivatives with a considerable amounts of the dehydro forms and the yields are probably lower.

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Asparagine appeared no problem regardless of its position in the peptide. However, glutamine appeared mainly as PTH-glutamic acid.

The use of the conc. HCl to split the acylserine and acyl-threonine has been successful in confirming the position of seryl and threonyl residues in the peptides.

The total residues as calculated from the sequence obtained in this experiment are in good accordance with the amino acid composition of cobrotoxin reported in the preceeding paper (1). The sequence as summarized from this study is elucidated in Fig. 4.

In comparison with the entire sequence of toxin α (3) and erabutoxin b (18) as illustrated in Fig. 5, 31 residues are found to be common to the three neurotoxins. These include the positions of all cystinyl residues which are important to the biological activity in these types. Moreover, the sequence of cobrotoxin shows very closely related to that of toxin α. There are only teen differences in amino acid composition of toxin α which express themselves in 12 different residues and one missing residue along the whole chain. Since both of the toxins are isolated from the same family of snake, the 13 variant residues are comparatively high from the evolutionary point of view.

In considering the relationship of the structure of cobrotoxin to its activity, a number of noteworthy features of the sequence may have important functions in maintaining the activity.

1. The basic amino acids and the functional residues are mainly concentrated in the middle part (position 25-40) of the sequence, leaving the remaining part very few basic residues. Thus, there are 2 lysyl residues out of the 3, 5 arginyl residues out of the 6, 1 histidyl residue out of the 2, and all aromatic functional residues, 2 tyrosyl and 1 tryptophyl, occured in this part. It must be noted that the two tyrosyl residues react readily in the native protein with iodine chloride (19), and also that the native protein loses its lethality after tryptic digestion (20). These all indicate that this part must expose outward on the protein and may play an important role in maintaining the toxicity.

2. The remaining part of the sequence contains mainly the hydrophilic residues. Remarkably, there are no hydrophobic residues appeared along the sequence from the position 2-24. This suggests that the hydrophobic environment is probably not required for maintaining the protein in its active configuration.

3. The acidic residues do not appear to show any particular distribution in the sequence and many of them are amidated.

These indicate that the intramolecular electrostatic bonds are also unimportant to keep in the active form.   Consequently, it seems likely that the disulfide bonds are probably the only bond to maintain the protein in its active configuration.   This is strikingly in accord with the fact that on the prolonged treatment with 8 M urea, cobrotoxin still retains fully its lethality (21).

V.   Conclusion

Reduced and S-carboxymethylated cobrotoxin was digested with trypsin and chymotrypsin.   Nine peptides were isolated from the tryptic digest by a combination of electrophoresis and chromatography on paper.   The amino acid sequence in these peptides was determined by both the application of the Edman degradation and the use of proteolytic enzymes, leucine aminopeptidase and carboxypeptidases.   The arrangement of nine peptides into a linear structure was made by comparing the amino acid compositions of the overlapping peptides isolated from chymotryptic digest of RCM-cobrotoxin.   The striking distributional regularities of the basic and hydrophilic residues in the sequence, and the importance of the positions of half-cystinyl residues which form the disulfide bonds for maintaining the protein in its active configuration are considered in connection with structure-activity relations of the protein.

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Table I

Amino acid composition of tryptic peptides from RCM-cobrotoxin

Peptides	Amino acid composition	Total residues	Yield %
T-1	$(CM\text{-}cys_{0.8}; Asp_{2.0})$	3	22
T-2	$(CM\text{-}cys_{1.5}; Asp_{2.9}; Thr_{2.0}; Glu_{1.0}; Gly_{1.1}; Ile_{2.0})\text{-}Arg_{1.1}$	12	18
T-3	$(CM\text{-}cys_{2.5}; Asp_{1.9}; Thr_{4.7}; Ser_{2.8}; Glu_{5.0}; Pro_{1.1}; Gly_{3.0}; Leu_{1.0}; Tyr_{0.9}; His_{1.0})\text{-}Lys_{1.0}$	26	29
T-4	$(CM\text{-}cys_{1.9}; Ser_{0.9}; Pro_{1.1}; Gly_{2.0}; Val_{0.9})\text{-}Lys_{1.0}$	8	29
T-5	$(Thr_{1.0}; Glu_{1.0})\text{-}Arg_{1.0}$	3	36
T-6	$(Asp_{1.0}; His_{1.0})\text{-}Arg_{1.0}$	3	31
T-7	$(Gly_{0.9}; Tyr_{0.3})\text{-}Arg_{1.0}$	3	30
T-8	$Trp_{0.9}\text{-}Arg_{1.0}$	2	27
T-9	$Lys_{1.1}; Arg_{1.0}$	2	28
T-10	$(CM\text{-}cys_{2.3}; Asp_{5.1}; Thr_{1.9}; Glu_{1.0}; Gly_{1.3}; Ile_{2.1}; Arg_{1.0})$	15	5.3
T-11	$(CM\text{-}cys_{2.7}; Asp_{1.8}; Thr_{4.8}; Ser_{2.7}; Glu_{5.0}; Pro_{0.9}; Gly_{3.0}; Leu_{0.9}; Tyr_{0.8}; His_{1.0})$	25	3.2
T-12	$Gly_{1.0}; Tyr_{0.9}$	2	2.0
T-13	$(Asp_{1.0}; His_{1.0}; Trp_{0.9}; Arg_{2.1})$	5	2.0
T-14	Arg	1	2.0
T-15	Lys	1	3.0

Table II

Amino acid composit:on of chymotryptic peptides from tryptic peptide T-3

Peptides	Amino acid composition	Total residues
T-3-C-1	$(CM\text{-}cys_{1.3};\ Asp_{1.0};\ Thr_{1.0};\ Ser_{1.0};\ Glu_{1.0};$ $Gly_{3.2})\text{-}Tyr_{0.7}$	10
T-3-C-2	$(CM\text{-}cys_{0.8};\ Asp_{1.0};\ Glu_{2.9};\ Leu_{1.0};\ His_{1.0})$	7
T-3-C-3	$(Thr_{2.1};\ Ser_{1.9};\ Glu_{1.0};\ Pro_{1.0})$	6
T-3-C-4	$Thr_{2.0}$	2
T-3-C-5	Lys	1

Table III

Amino acid composition of chymotryptic peptides from RCM-cobrotoxin

Peptides	Amino acid composition	Total residues	Yield %
C-1	$CM\text{-}cys_{2.3}$; $Asp_{3.0}$; $Thr_{1.7}$; $Arg_{1.0}$	9	12.1
C-2	$(CM\text{-}cys_{1.3}$; $Asp_{1.4}$; $Thr_{1.0}$; $Ser_{0.9}$; $Glu_{1.2}$; $Gly_{3.0})\text{-}Tyr_{0.8}$	10	8.9
C-3	$CM\text{-}cys_{2.1}$; $Asp_{3.0}$; $Thr_{1.8}$; $Glu_{1.3}$; $Gly_{1.2}$; $Ile_{2.0}$; $Arg_{0.6}$	13	5.2
C-4	$(CM\text{-}cys_{1.8}$; $Asp_{1.0}$; $Thr_{1.8}$; $Ser_{0.7}$; $Glu_{1.1}$; $Gly_{2.9})\text{-}Tyr_{0.7}$	11	4.0
C-5	$CM\text{-}cys_{0.6}\text{-}Tyr_{1.0}$	2	1.1
C-6	Asp	1	6.7
C-7	$CM\text{-}cys_{0.8}$; $Glu_{0.9}$; $Leu_{1.0}$; $His_{1.0}$	4	0.6
C-8	$Asp_{1.0}$; $Glu_{1.0}$; $Gly_{0.6}$; $Ile_{2.0}$	5	1.9
C-9	$CM\text{-}cys_{0.8}$; $Asp_{1.0}$; $Glu_{3.0}$; $Leu_{0.8}$; $His_{1.0}$	7	18.9
C-11	$CM\text{-}cys_{0.8}$; $Asp_{2.0}$; $Arg_{1.0}$	4	6.8
C-12	$Thr_{2.0}$; $Ser_{2.0}$; $Glu_{1.0}$; $Pro_{1.0}$	6	31.0
C-13	$CM\text{-}cys_{2.2}$; $Asp_{0.8}$; $Thr_{1.1}$; $Ser_{1.0}$; $Glu_{1.1}$; $Pro_{1.2}$; $Gly_{2.1}$; $Val_{1.0}$; $Lys_{1.0}$; $Arg_{2.0}$	13	9.1
C-14	$(Gly_{1.0}$; $Arg_{0.5})\text{-}Tyr_{0.8}$	3	7.6
C-15	$(Asp_{1.1}$; $Gly_{1.0}$; $His_{0.9}$; $Arg_{1.8})\text{-}Tyr_{0.4}$	6	6.0
C-16	$Asp_{1.0}$; $Lys_{2.0}$; $Arg_{1.9}$; Trp_{+}	6	2.0
C-17	$(Lys_{1.0}$; $Arg_{1.2})\text{-}Trp_{+}$	3	4.8
C-18	$(Lys_{2.0}$; $Arg_{1.3})\text{-}Trp_{+}$	4	5.8

Table IV

Probable sequence of chymotryptic peptides from RCM-cobrotoxin

Peptides	Probable amino acid sequence	Net charge
C-1	H-CyS-CyS-Thr-Thr-Asp-Arg-CyS-Asn-Asn-OH	-3
C-2	H-Gly-CyS-Ser-Gly-Gly-Glu-Thr-Asn-CyS-Tyr- "	-3
C-3	H-Gly-Ile-Glu-Ile-Asn-CyS-CyS-Thr-Thr-Asp-Arg-CyS-Asn-OH	-4
C-4	H-Thr-Gly-CyS-Ser-Gly-Gly-Glu-Thr-Asn-CyS-Tyr-OH	-3
C-5	H-CyS-Tyr-OH	-1
C-6	H-Asp-OH	-1
C-7	H-Leu-Glu-CyS-His-OH	-1
C-8	H-Gly-Ile-Glu-Ile-Asn-OH	-1
C-9	H-Leu-Glu-CyS-His-Asn-Gln-Gln-OH	-1
C-11	H-Arg-CyS-Asn-Asn-OH	0
C-12	H-Ser-Ser-Gln-Thr-Pro-Thr-OH	0
C-13	H-Arg-Thr-Glu-Arg-Gly-CyS-Gly-CyS-Pro-Ser-Val-Lys-Asn-OH	0
C-14	H-Arg-Gly-Tyr-OH	+1
C-15	H-Arg-Asp-His-Arg-Gly-Tyr-OH	+2
C-16	H-Lys-Lys-Arg-Trp-Arg-Asp-OH	+3
C-17	H-Lys-Arg-Trp-OH	+2
C-18	H-Lys-Lys-Arg-Trp-OH	+3
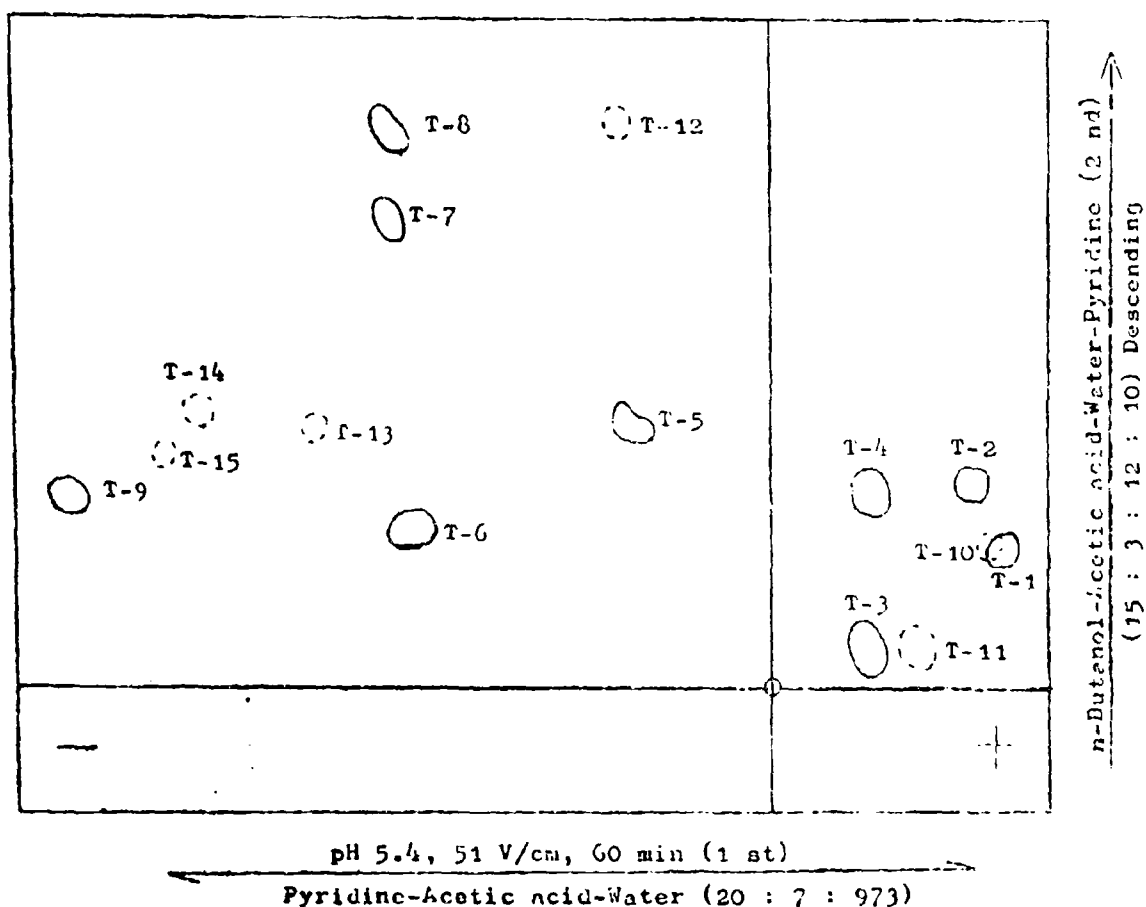
Fig. 1.   Peptide map of the tryptic hydrolysate of RCM-cobrotoxin.

One mg. of the hydrolysate was used on a sheet of Whatman 3 MM
paper (74 x 56 cm).   The electrophoresis was carried out at pH
5.4 in pyridine-acetic acid-water (20 : 7 : 973, v/v) for 60 min
at 51 V/cm.   Chromatography was run with n-butanol-acetic acid-
water-pyridine (15 : 3 : 12 : 10, v/v) by the descending method
for 18 h.
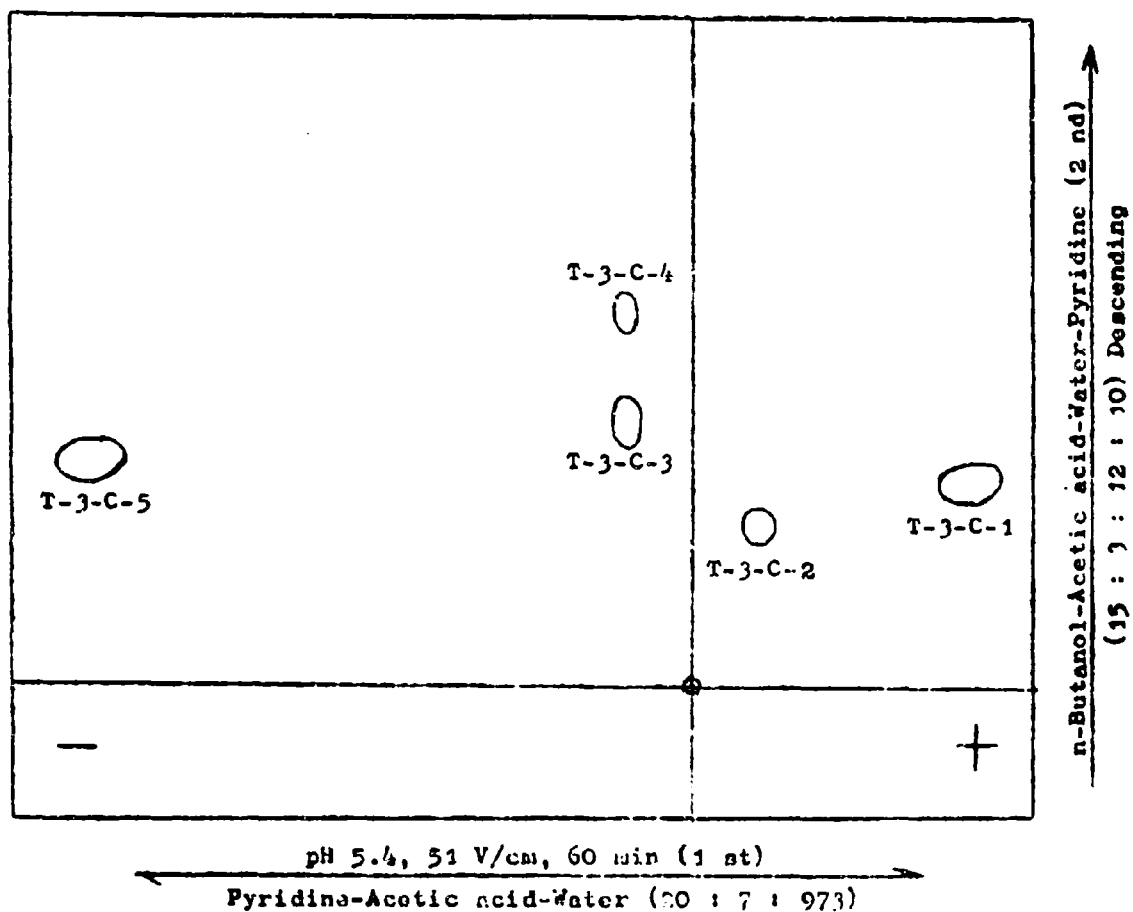
Fig. 2. Peptide map of the chymotryptic hydrolysate of tryptic peptide T-3.

Conditions for preparation of the map were the same as those in Fig. 1.
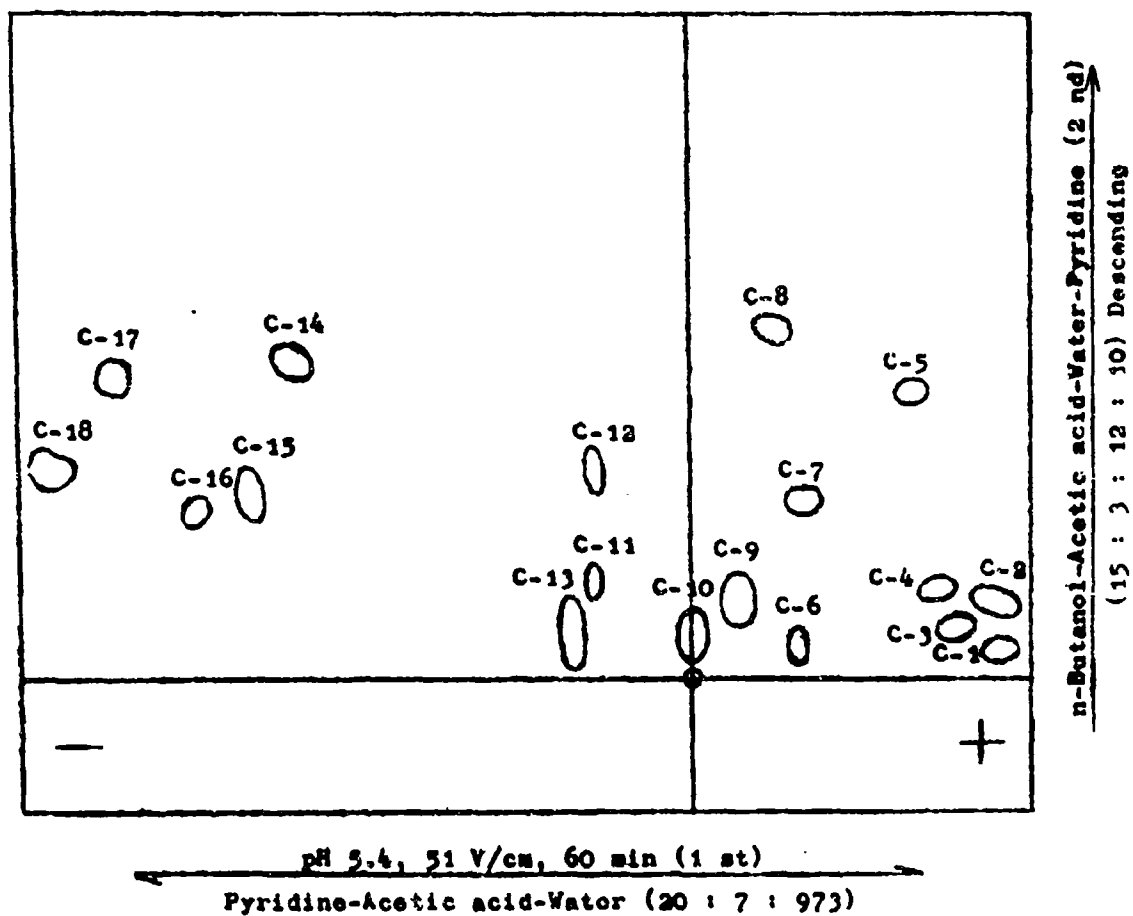
Fig. 3.   Peptide map of the chymotryptic hydrolysate of RCM-cobrotoxin.

Conditions for preparation of the map were the same as those in Fig. 1.

T-3, T-11

T-3-C-3

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |

H-Leu-Glu-CyS-His-Asn-Gln-Gln-Ser-Ser-Gln-Thr-Pro-Thr-Thr-Thr-

C-7

C-9

C-12

C-4

T-3

T-11

T-3-C-1

T-14 T-9 T-13

T-3-C-5 T-8

| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |

Gly-CyS-Ser-Gly-Gly-Glu-Thr-Asn-CyS-Tyr-Lys-Lys-Arg-Trp-Arg-

C-5

C-17

C-2

C-18 C-15

C-4

C-16

T-13 T-7

T-6 T-12 T-15 T-5 T-4

| 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 |

Asp-His-Arg-Gly-Tyr-Arg-Thr-Glu-Arg-Gly-CyS-Gly-CyS-Pro-Ser-Val-Lys-

T-16 C-14 C-13

C-15

T-10

T-2 T-1

| 48 | 49 | 50 | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 |

Asn-Gly-Ile-Glu-Ile-Asn-CyS-CyS-Thr-Thr-Asp-Arg-CyS-Asn-Asn-OH

C-13 C-8 C-11

C-1

C-3

Fig. 4. Amino acid sequence of RCM-cobrotoxin.

The positions and designations of the peptides that are discussed in the text are given.

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APPENDIX B-5

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Cobrotoxin	H-Leu-Glu-Cys-His-Asn-Gln-Gln-Ser-Ser-Gln-Thr-Pro-Thr-Thr-														Thr-Gly-Cys-Ser-Gly-Gly-					
Toxin α	H-Leu-Glu-Cys-His-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Pro-Thr-Thr-														Lys-Thr-Cys-Pro-Gly-					
Erabutoxin b	H-Arg-Ile-Cys-Phe-Asn-His-Glu-Ser-Ser-Glu-Pro-Thr-Thr-Trp-Lys-Thr-Cys-Pro-																			

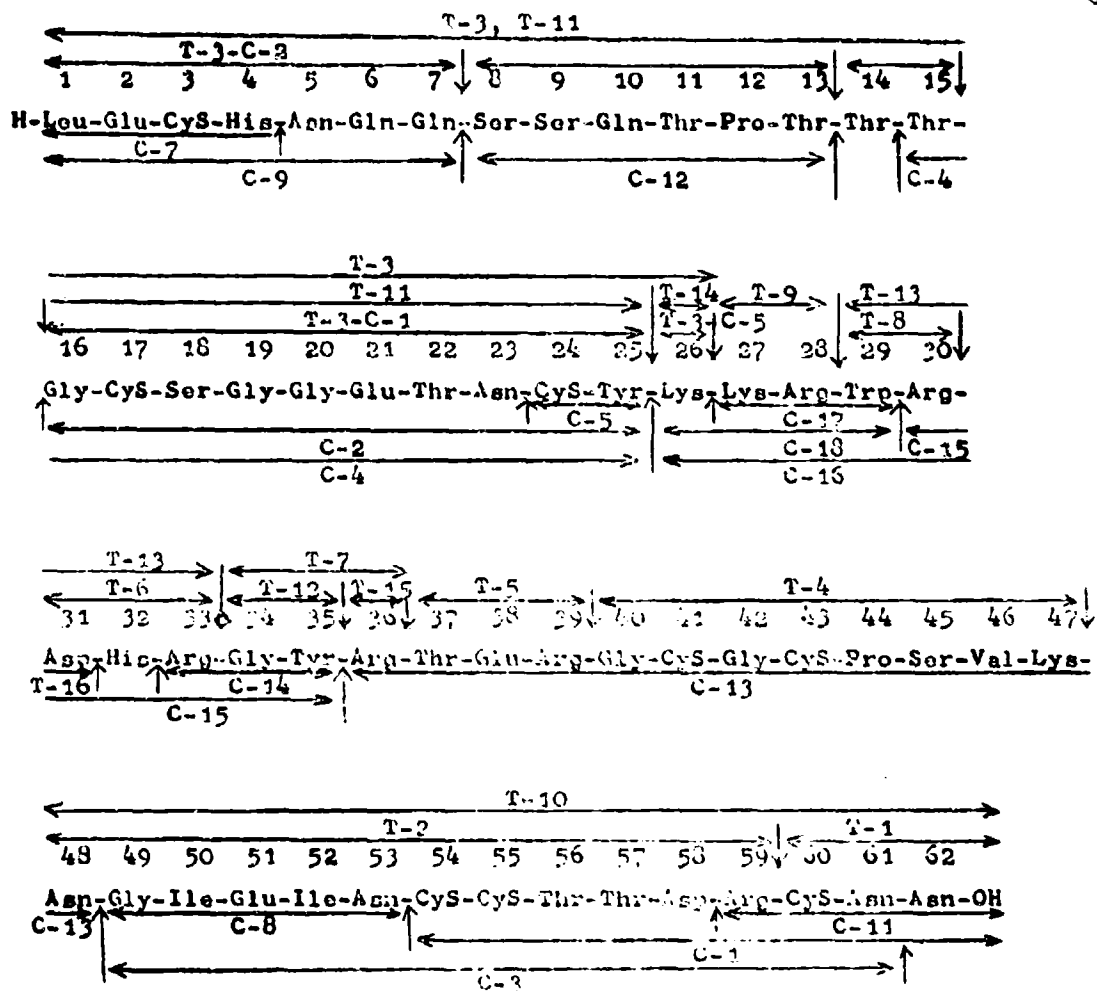|	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
	Glu-Thr-Asn-Cys-Tyr-Lys-Lys-Arg-Trp-Arg-Asp-His-Arg-Gly-Tyr-Arg-Thr-Glu-Arg-Gly-Cys-Gly-Cys-																						
	Glu-Thr-Asn-Cys-Tyr-Lys-Lys-Val-Trp-Arg-Asp-His-Arg-Gly-Thr-Ile-Ile-Glu-Arg-Gly-Cys-Gly-Cys-																						
	(Glu-Ser-His-Cys-Tyr-Ser-Gly)Lys-Glu(Ser)Asp-Phe-Arg-Gly-Thr-Ile-Ile-Glu-Arg-Gly-Cys-Gly-Cys-																						

	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
	Pro-Ser-Val-Lys-Asn-Gly-Ile-Glu-Ile-Asn-Cys-Cys-Thr-Thr-Asp-Arg-Cys-Asn-Asn-OH																		
	Pro-Thr-Val-Lys-Pro-Gly-Ile-Lys-Leu-Asn-Cys-Cys-Thr-Thr-Asp-Lys-Cys-Asn-Asn-OH																		
	(Pro-Thr-Val-Lys-Pro-Gly-Leu-Lys-Ile-Asn-Cys-Cys-Ser-Glu-Val-Ser)Cys-Glu-Asn-OH																		

Fig. 5. Amino acid sequence of neurotoxins.

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List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to
   lethality.   Biochim. Biophys. Acta, 133 (1967) 346.

2. Optical rotatory dispersion of cobrotoxin.
                       J. Biochem., 61 (1967) 272.

3. Biochemical studies on the toxic nature of snake venom.
                       Intern. Conger. Biochem., 7th, Tokyo, Col.
                       VIII, 1 (1967).

4. Studies on fluorescent cobrotoxin.
                       Biochim. Biophys. Acta, 147 (1967) 600.

5. Study on I^{131} labeled cobrotoxin.
                       Toxicon, 5 (1968) 295.

6. Optical rotatory dispersion and circular dichroism of cobrotoxin.
                       Biochim. Biophys. Acta, 168 (1968) 373.

7. Amino acid composition and end group analysis of cobrotoxin.
                       Toxicon, 6 (1969)   .

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DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)	2a. REPORT SECURITY CLASSIFICATION
Department of Biochemistry Kaohsiung Medical College Kaohsiung, Taiwan	Unclassified
	2b. GROUP

3. REPORT TITLE

BIOCHEMICAL STUDIES ON THE TOXIC NATURE OF SNAKE VENOM (U)

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final Report No. 6. June 1968 - June 1969

5. AUTHOR(S) (First name, middle initial, last name)

Chen-chung Yang

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
14 July 1969	23	21

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
DA-CRD-AFE-S92-544-68-G124	
b. PROJECT NO. 2N061102B71D	J-262-6
c. Task 00 063FE	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. DISTRIBUTION STATEMENT

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army R&D Group (Far East) APO San Francisco 96343

13. ABSTRACT

Studies on the chemical properties of cobrotoxin have established its amino acid composition, and revealed that the toxin has a single peptide chain of 62 amino acids cross-linked by 4 disulfide bonds with amino terminal leucine and carboxyl terminal asparagine. In comparison with the entire sequence of toxin α from the venom of Naja nigricollis the similarities obviously must lie in its alike chemical structure. Knowledge of the sequence of cobrotoxin is required in order to understand the location of the essential amino acid residues required for the maintenance of the active conformation of this type. It is the purpose of this experiment to present the sequence of amino acids in cobrotoxin.

Reduced and S-carboxymethylated cobrotoxin was digested with trypsin and chymotrypsin. Nine peptides were isolated from the tryptic digest by a combination of electrophoresis and chromatography on paper. The amino acid sequence in these peptides was determined by both the application of the Edman degradation and the use of proteolytic enzymes, leucine aminopeptidase and carboxypeptidases. The arrangement of nine peptides into a linear structure was made by comparing the amino acid compositions of the overlapping peptides isolated from chymotryptic digest of RCM-cobrotoxin. The striking distributional regularities of the basic and hydrophilic residues in the sequence, and the importance of the positions of half-cystinyl residues which form the disulfide bonds for maintaining the protein in its active configuration are considered in connection with structure-activity relations of the protein.

(Cont'd)

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Unclassified
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Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Snake Venom Cobrotoxin Amino acid Analysis Trypsin Peptide Formosa						

Unclassified
Security Classification

In comparison with the entire sequence of toxin α and erabutoxin b, 31 residues are found to be common to the three neurotoxins. It is noteworthy that all cystinyl residues which are important to the biological activity in neurotoxin are all in the same position, and leave the center sequence from residue 25-40 free. It is speculated that this central non-crosslinked sequence containing most of the basic and aromatic amino acids in close order might be the "active site" of the neurotoxin molecule. Moreover, the sequence of cobrotoxin shows very closely related to that of toxin α isolated from the same family of snake. There are only teen differences in amino acid composition of toxin α which express themselves in 12 different residues and one missing residue along the whole chain. In view of the general similarity among neurotoxins isolated from different snake venoms, investigations of the structural differences among them should reveal a good deal about the structural features responsible for the venom toxicity. (Author)